Pflügers Arch (1983) 396:308–314

Pflügers Archiv
European Journal of Physiology

Evidence for Electroneutral Sodium Chloride Cotransport in the Cortical Thick Ascending Limb of Henle's Loop of Rabbit Kidney*

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Abstract. Previously we have shown that chloride reabsorption in the isolated perfused cortical thick ascending limb of Henle's loop of rabbit (cTAL) is dependent on the presence of sodium and potassium. The present study was performed to elucidate the dependence on chloride in quantitative terms. Ninety-four cTAL segments were perfused at high rates with solutions of varying chloride concentration. Chloride was substituted by sulfate, methylsulfate or nitrate. The open circuit transepithelial electrical potential difference (PD_{te}, mV) and the specific transepithelial resistance (R_t, Ωcm^2) were measured, and from both the equivalent short circuit current (I_{sc}) was calculated. The correlation of I_{sc} versus Cl^- concentration (in 294 observations) revealed a saturation kinetics depending on the $(Cl^-)^2$ concentration. The apparent constants were $K_{1/2}$ 50 mmol $\cdot l^{-1}$ and $I_{sc,max}$ 198 μA cm^{-2}. These results are compatible with the assumption of 2 Cl^- interacting with the luminal cotransport system. Although this finding in conjunction to our previous observations already is highly suggestive for a non-charged carrier this question was pursued further by recording the membrane *PD* across both cell membranes during luminal application of furosemide. The data ($n = 26$) indicate that furosemide (10^{-5} mol $\cdot l^{-1}$, lumen) produces an immediate decline in PD_{te} to values close to zero, and a simultaneous hyperpolarization of both cell membranes by 15 ± 2 mV for the basolateral and by 8 ± 2 mV for the lumen membrane. These data exclude the possibility that the lumen positive transepithelial *PD* is generated by a net negative current flow from the lumen into the cell. It is concluded that the cotransport across the luminal membrane of the cTAL segment is electroneutral and involves 1 Na^+:2 Cl^-:1 K^+. The hyperpolarization of both cell membranes observed immediately after application of furosemide is caused most likely by a rapid fall in intracellular chloride activity.

Key words: Thick ascending limb of Henle's loop – Rabbit – Furosemide – Loop diuretics – Na-Cl_2-K cotransport – Isolated perfused tubule

Introduction

Previously we have shown that in sharp contrast to earlier observations by others [5, 25, 32] active transport of chloride in the thick ascending limb of the loop of Henle (TAL) is dependent on the presence of sodium [12]. Meanwhile this observation has been confirmed for the mouse TAL segment [23], and for the diluting segment of amphiuma [31], trout [28] and salamander [33]. On the basis of these data, and in conjunction with the observation that chloride reabsorption in the TAL segment is inhibited by ouabain [5, 14, 32], we have previously concluded that sodium and chloride are cotransported in this nephron segment, and that the driving force for this process is provided by the sodium gradient across the luminal membrane which in turn is maintained by the basolateral $(Na^+ + K^+)$-ATPase. Recently, we have reported that the equivalent short-circuit-current is dependent also on the presence of luminal potassium [16]. This finding prompts the question as to the stoichiometry of the cotransporter for the three ions Na^+, K^+, and Cl^-. Furthermore, so far no explanation can be given for the mechanisms responsible for the lumen positive transepithelial electrical potential difference (PD_{te}) which is a constant finding in the TAL segment and in the amphibian diluting segment when perfused at high rates, such that level flow conditions prevail, with identical control solutions on both sides of the epithelium. It was tempting to speculate that this *PD* could be generated by the carrier itself if it were negatively charged [14]. Thus, the present study attempts to define the chloride stoichiometry by correlating active transport to the chloride concentration on both sides of the epithelium. In a second set of experiments, the *PD* transients of the basolateral membrane and across the entire epithelium, observed after rapid inhibition by furosemide, are used to decide whether the carrier is negatively charged, and thus responsible for the lumen positive transepithelial *PD*. The data indicate that possibly 2 Cl^- interact with the carrier. The carrier most likely is not negatively charged, and thus is not responsible for the lumen positive PD_{te} in the rabbit cortical thick ascending limb segment (cTAL).

Methods

Female white New Zealand rabbits (body weight: 500–800 g) ($n = 121$) were used in the present study. The Burg method to dissect and to perfuse isolated nephron segments was employed [6]. The modifications as they are customary in our laboratory have been described elsewhere [11, 15]. On average a suitable cTAL segment with a length of 200–1000 μm was obtained within 5 min after sacrifice of the animal. Segments were transferred in the perfusion chamber, cannulated and perfused at high rate (10–20 nl $\cdot$ min^{-1}). Typically the experiment was begun only a few minutes after successful dissection. All experiments were performed at 38° C. Bath temperature and the temperature of the bath

* The study was supported by: Deutsche Forschungsgemeinschaft, Gr. 480/4, 480/5, 480/6-2. Parts of this study have been presented at the Int. Symp. Ototoxicity, Reinhardshausen, April 1981, at the 54th Meeting Deutsche Physiol. Ges. 1981, and at the 14th Meeting Americ. Soc. Nephrol. 1981
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Table 1. Composition of solutions. All concentrations in mmol · l^{-1}

Cl^-	0	2	10	30	50	75	100	150	300 Na	300 Cho
SO_4^{2-} [a]	75	74	70	60	50	38	25	0	0	0
$CH_3SO_4^-$ [a]	150	148	140	120	100	75	50	0	0	0
NO_3^- [a]	150	148	140	120	100	75	50	0	0	0
Gluconate$^-$	8	8	8	8	8	4	3	2.6	2.6	2.6
$H_2PO_4^-$	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
HPO_4^{2-}	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Na^+	150	150	150	150	150	150	150	150	300	150
K^+	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Ca^{2+} [c]	4	4	4	4	4	2	1.5	1.3	1.3	1.3
Choline$^+$	0	0	0	0	0	0	0	0	0	150
Glucose	5	5	5	5	5	5	5	5	5	5
Mannitol [b]	75	74	70	60	50	37	25	0	0	0

[a] Alternatively sulfate, methylsulfate, or nitrate were used as substituting anions

[b] Sulfate solutions were made isotonic by adding mannitol

[c] In the presence of large concentration of sulfate (methylsulfate, nitrate) Ca^{2+} was increased to maintain a reasonably constant ionized calcium concentration. In separate experiments it was shown that e. g. at 50 Cl^- variation of Ca^{2+} between 1 und 5 mmol · l^{-1} had no influence upon the measured I_{sc}

influx were kept constant with a dual channel feed back system (W. Hampel, Frankfurt, FRG). This system enables one to vary bath perfusion between 0 and 60 ml · min^{-1} without disturbing constancy of bath temperature. Transepithelial electrical potential difference (PD_{te}) was measured continuously on both ends of the perfused segment [11]. Specific transepithelial resistance (R_t) was obtained from the input resistance observed after injection of short current pulses (20 nA, 600 – 800 ms) as described previously [11]. The system was, however, modified slightly by inclusion of a stimulation isolation unit (Grass Instruments, Quincy, MA, USA). Since the injected current in this system returns to the unit via a separate agar bridge connected to an Ag/AgCl electrode, this circuit is off ground, and, thus, no correction for a current pulse induced voltage deflection across the resistances of the agar bridge and the calomel electrode, which connect the bath solution to ground, are necessary.

From both values R_t and PD_{te} the equivalent short circuit current (I_{sc}) was calculated:

$$I_{sc} = \frac{PD_{te}}{R_t} (\mu A \cdot cm^{-2}).$$

It should be kept in mind that this I_{sc} is not a measured current when PD_{te} is clamped to zero. The use of I_{sc} as a quantification of NaCl transport across the cTAL segment makes the important implication that no other ion movements such as e.g. H^+ secretion contribute to the lumen positive PD_{te}. We shall deal with this point in the discussion section.

The details of the measurements of the electrical *PD* across the basolateral membrane are provided in a subsequent report [17]. Briefly, KCl filled electrodes with a resistance of 80 – 100 MΩ were used to impale the basolateral membrane of a perfused cTAL segment with the aid of a piezo stepper (Physik Instr., Waldbronn, FRG). The electrode was connected through an Ag/AgCl half cell to an electrometer (FD 223, WPI, Science Trading, Frankfurt, FRG) and PD_{bl} was measured with reference to the grounded bath electrode.

Series 1 comprizes 94 cTAL segments in which the equivalent short circuit current [$I_{sc} = PD_{te}/R_t$ (A · cm^{-2})] was measured at different chloride concentrations. In randomized fashion the experiment was begun at nominally zero or at 150 mmol · l^{-1} Cl^- concentration on both sides. Then, lumen and bath perfusates were changed to another Cl^- concentration (2, 10, 30, 50, 75, 100, 150 or 300 mmol · l^{-1}, Table 1). In some of the tubules it was possible to test 5 or more different concentrations. A tubule was discarded from the study if only measurements at one single concentration were obtained. The I_{sc} at 150 mmol · l^{-1} Cl^- was used as the reference measurement in each tubule, and was recorded at least twice, on average four times, for each segment. The experiment was terminated if the results at 150 mmol · l^{-1} deviated from the preceding observations by more than 20 %. The means for each individual segment were used for further calculations.

In a second type of experiment (14 observations in 2 tubules) the time course of PD_{te} was recorded after application of furosemide ($1 - 5 \cdot 10^{-5}$ mol · l^{-1}, lumen). For these and the subsequent experiments the perfusion system had to be modified slightly from the one described previously [11]. Lumen perfusion was carried out through a dual channel perfusion pipette made from theta glass (Hilgenberg, Malsfeld, FRG). One channel was filled with control solution through a fluid exchange pipette. The other channel was preloaded with the furosemide solution through a separate fluid exchange catheter (comp. Fig. 1 of a subsequent report [17]). The hydrostatic pressure was put on either channel by a pneumatically controlled 3-way valve (Altex, Abimed, Düsseldorf, FRG). This set-up enables one to perform extremely rapid changes of the lumen perfusate. From concentration step experiments we know that the change in lumen perfusates is complete within less than 0.3 – 3.0 s. In this series no attempt was made to record absolute values of transepithelial resistance. Instead, current pulses were applied at approximately 7 Hz to monitor possible rapid changes in transepithelial resistance.

In series 3, 26 tubules were studied. The methodological approach was identical to that of series 2. In addition to the PD_{te} basolateral electrical potential difference was recorded as described briefly above, and as reported explicitely elsewhere [17].

The composition of the used solutions is given in Table 1. All solutions were gassed with oxygen. pH and electrolyte composition of the solutions used were checked repeatedly using standard methods.

Data are presented as original recordings for individual tubules or as mean ±SEM. In series 1 a computer-aided fitting procedure was used to obtain a least square fit to the Michaelis-Menten equation:

$$I_{sc} = \frac{I_{sc,max} \cdot (Cl^-)^n}{(K_{1/2})^n + (Cl^-)^n} - I_{sc,o}.$$

In this formula I_{sc} is the observed, $I_{sc,max}$ the maximal, and $I_{sc,o}$ the apparently Cl^- independent equivalent short circuit current. $K_{1/2}$ is the apparent affinity constant, and n is the number of Cl^- ions interacting with the carrier. The three parameters $I_{sc,o}$, $I_{sc,max}$, and $K_{1/2}$ were fitted to the data. The parameters were varied in discrete decrements of 5%, and a least square fit for the three parameters was obtained. During this step always one of the data points ($n = 294$) was omitted, and thus the standard deviations for the least square mean values of $I_{sc,o}$, $I_{sc,max}$, and $K_{1/2}$ were obtained. This procedure was carried out three times: For the assumption than n equals 1, 2, or 3. Then the sums of the squares of the residuals for the fit to the 3 models ($n = 1$, $n = 2$, and $n = 3$) were compared statistically. A P value of < 0.05 was accepted as an indication for statistical significance throughout this study.

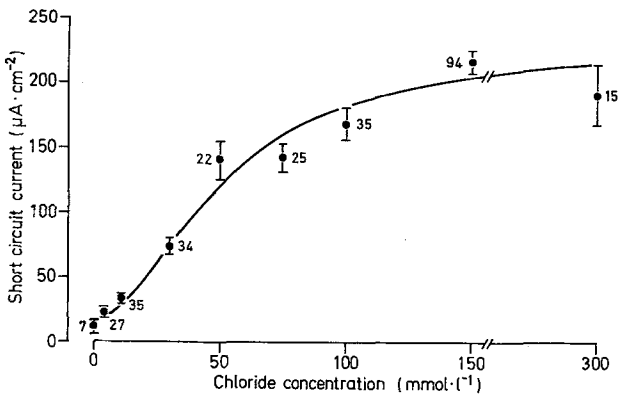

Fig. 1. Chloride dependence of equivalent short circuit current in isolated perfused cTAL segments. The equivalent short circuit current (PD_{te}/R_t, μA · cm^{-2}) is plotted versus the chloride concentration on both sides of the epithelium. The concentration of Na^+ was kept constant at 150 mmol · l^{-1} (except sol. "300 Na", Table 1). The solid curve is calculated from a least square fit to the Michaelis-Menten equation, correlating the equivalent short circuit current to $\{Cl^-\}^2$. This fit is statistically significantly superior to the alternatively tested correlations: equivalent short circuit current versus $\{Cl^-\}^1$, and versus $\{Cl^-\}^3$. Besides each mean value the number of tubules is given

Results

The results of series 1 are depicted in Fig. 1. As the solutions used cannot be identical in either total or in ionized calcium, pilot experiments were performed to test whether changes in calcium have any influence on I_{sc} at high sulfate concentrations. Thus it was shown that e. g. at Cl^- 30, and SO_4^{2-} 60 variation of total Ca^{2+} between 1 and 5 mmol · l^{-1} had no detectable influence on the measured I_{sc}. Comparison of the data obtained with either of the substituting anions revealed no systematic difference. Consequently, the data were pooled. Similarly, no difference was apparent for the data obtained with 300 Na^+ and those obtained with 150 Na^+ + 150 choline$^+$. Finally, it was shown in 4 tubules that the increase in osmolality, as was present in the experiments utilizing 300 Na^+ or 300 choline$^+$ solutions, and which amounted to 300 mosm · l^{-1} had only a small, yet statistically significant effect per se on the I_{sc} (ΔI_{sc}: −18 ± 5%). This was demonstrated by comparing the I_{sc} at 150 Cl^- to that in a solution containing the identical concentrations but in addition 300 mmol · l^{-1} mannitol. As apparent from Fig. 1, I_{sc} increases with increasing Cl^- concentration. Table 2 indicates that the data are fitted best by a sigmoid function which was obtained from a least square fit to the Michaelis-Menten equation on the basis of a $\{Cl^-\}^2$ correlation. Using this approach the fit was significantly better than that to $\{Cl^-\}^1$ or $\{Cl^-\}^3$.

The apparent kinetic constants for the $\{Cl^-\}^2$ model are: $K_{1/2} = 49 \pm 31$ mmol · l^{-1}, $I_{sc,max}$ 198 ± 10 μA · cm^{-2}, and $I_{sc,o}$ 23 ± 12 μA · cm^{-2}.

A typical tracing of series 2 is shown in Fig. 2. Apparently, furosemide causes an immediate fall in PD_{te}. The time constant of this PD drop is difficult to quantify as the tracing may still be biased by the speed with which the fluid exchange becomes effective. It appears safe to conclude that the halftime for the decay in PD_{te} was less than 0.4 s. On other occasions (Fig. 3) the fall in PD_{te} was slower, and this most likely is caused by a slower change of perfusates. The mean half value time in 14 observations, was 0.98 ± 0.14 s. The fall in PD_{te} was complete within 1–2 s, and the mean PD_{te} after

Table 2. Apparent kinetic constants for the least square fit to the Michaelis-Menten equation of the data depicted in Fig. 1. $K_{1/2}$ = half maximal concentration for Cl^- · $I_{sc,o}$ = y-axis intercept. $I_{sc,max}$ = maximal short circuit current. Note that the SEMs are smallest for $\{Cl^-\}^2$. Also the sums of the squares of the residuals are significantly smaller for $\{Cl^-\}^2$ when compared to $\{Cl^-\}^1$ or to $\{Cl^-\}^3$

	$K_{1/2}$ (mmol · l^{-1})	$I_{sc,o}$ (μA · cm^{-2})	$I_{sc,max}$ (μA · cm^{-2})
$\{Cl^-\}^1$	85 ± 56	285 ± 33	17 ± 35
$\{Cl^-\}^2$	49 ± 31	198 ± 10	23 ± 12
$\{Cl^-\}^3$	49 ± 36	177 ± 62	30 ± 33

furosemide was +0.9 ± 0.1 mV. The current induced voltage deflections increased slightly (+12%) and significantly after furosemide indicating a minute increase in input- and thus transepithelial resistance. Here again, quantification in terms of absolute changes in R_t is not possible: To obtain a high time resolution for the relative R_t changes frequent (7 Hz) and short (0.07 s) pulses had to be used. Therefore, the shape and the height of the pulse are already falsified by the pen recorder (3 dB frequency 3 Hz). In a qualitative way the present data confirm the previous finding of a small increase in transepithelial resistance after furosemide [7, 14]. Because the observed changes are small we cannot be certain as to when this change in resistance occurs. Close inspection of Fig. 2 indicates that the small increase in resistance builds up during the first few seconds. The results of series 3 are summarized in Fig. 3 and Fig. 4. Figure 3 is an original recording of a typical experiment, and Fig. 4 summarizes the PD_{bl} data of the entire series. It becomes apparent that from a baseline PD_{bl} of −62 ± 2 mV furosemide caused a hyperpolarization by 15 ± 2 mV. The time course of this hyperpolarization matches that of the concomitant fall in PD_{te}. From PD_{bl} and PD_{te} the lumen membrane PD (PD_l) can be calculated. Under control conditions PD_l was 68 ± 2 mV, and hyperpolarized to 76 ±

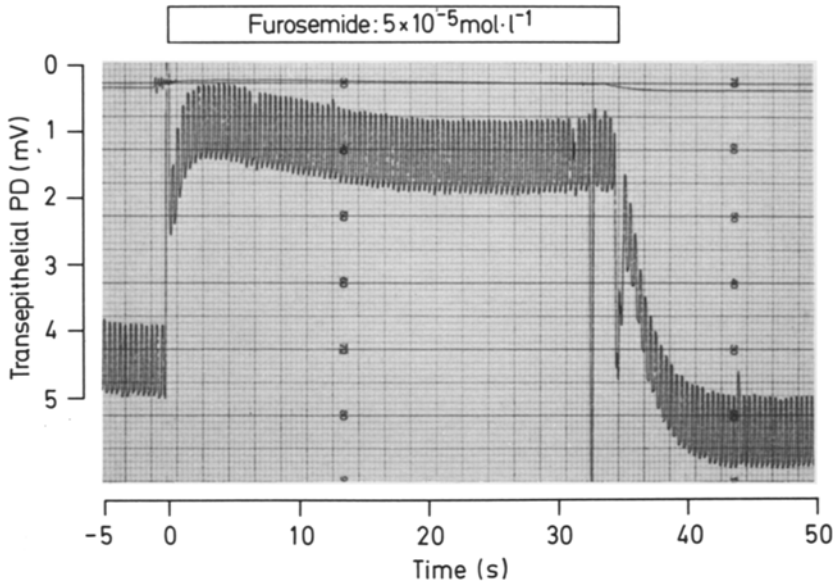

Fig. 2
A typical recording of PD_{te} after sudden change in the lumen perfusate to a furosemide containing solution. At zero time the perfusate is changed. The movement of the valve is easily recognized as the artifact (spike in upward direction at zero time). During the entire experiment hyperpolarizing current pulses of 2 nA, duration 0.07 s, frequency 7 Hz are injected to obtain a qualitative estimate of possible alterations in transepithelial resistance. The upper bound of the tracing represents the spontaneous PD_{te}. Note the very rapid fall in PD_{te} after furosemide. The current induced voltage deflection, and thus the transepithelial resistance, increase very slightly (control voltage deflection 1.03 mV, after furosemide 1.10 mV). After switching back to the control solution (downward spike at 33 s) the PD_{te} recovers very rapidly

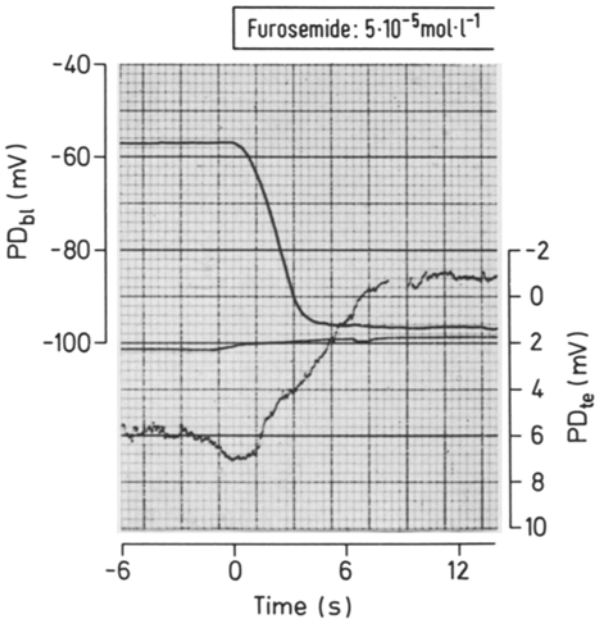

Fig. 3. A typical recording of the PD across the basolateral membrane (PD_{bl}) and of PD_{te} after addition of furosemide to the lumen perfusate. Note the marked hyperpolarization of PD_{bl} and the simultaneous fall in PD_{te}. The hyperpolarization of PD_{bl} (Δ 39 mV) exceeds the change in PD_{te} (Δ 7 mV). Therefore, the lumen membrane hyperpolarizes also (Δ 32 mV). The trace in the middle corresponds to the voltage recorded at the collection side of the tubule at lower sensitivity of the amplifier

2 mV after furosemide. In a few of these experiments the site of impalement was sufficiently close to the perfusion pipette, such that the current pulse induced voltage deflection across the basolateral membrane could be recorded. From this voltage deflection and the transepithelial voltage deflection the voltage divider ratio (VDR) can be obtained. The VDR, here defined as current pulse induced voltage deflection across the lumen membrane divided by that across the basolateral membrane, corresponds to the ratio of lumen and basolateral membrane resistances. The details of the VDR determination will be given in a subsequent report [17]. As

Table 3. Effect of furosemide ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1}) added to the lumen perfusate on the voltage divider ratio (VDR)

	Control	Furosemide	Control
VDR	2.8	1.5[a]	3.0
±SEM	0.5	0.2	0.5
n	7	7	6

[a] Significantly different from control values

shown in Table 3, VDR decreased significantly from a control value of 2.8 ± 0.5 to 1.5 ± 0.2 when furosemide was added to the lumen perfusate. This decrease in VDR was fully reversible when furosemide was removed.

Discussion

Dependence of I_{sc} on the Cl^- Concentration

The present investigation is an attempt to define the stoichiometry of the cotransport system present in the lumen membrane of the cTAL segment. The approach used, of varying the Cl^--concentration on both sides of the tubule epithelium and of recording the equivalent short circuit current, may be subject to several possible pitfalls: 1. The measured parameters R_t and PD_{te}, and thus I_{sc} have to correspond to the magnitude of secondarily active Cl^- transport across this epithelium. This, in fact, seems the case for the TAL segment as all manœuvres that reduce the PD_{te} at zero concentration difference for any ion across the epithelium, parallel the inhibition of salt transport [4, 36]. Clearly, no statement can be made about a coefficient linking I_{sc} and net Cl^- transport. It is, however, worth noting that both are in the same order of magnitude [4]. Furthermore, we do have experimental evidence that NaCl cotransport is the main transport event in the rabbit cTAL segment. Recent investigations [20, 34] have shown that this nephron segment does not transport HCO_3^- nor H^+. Also, the cTAL segment apparently has no rheogenic transport systems for organic substances such as glucose and amino acids. The I_{sc}, there-

fore, seems to be proportional to the rate of active NaCl reabsorption. On the other hand, the analysis of the data depicted in Fig. 1 revealed a small yet statistically significant $I_{sc,o}$ of 23 $\mu A \cdot cm^{-2}$ in the apparent absence of Cl^-. We do not know whether this small current is caused by a mechanism other than NaCl reabsorption or whether it reflects a small asymmetry in our measuring circuit which would amount to <0.7 mV. 2. To obtain the apparent kinetics a variety of solutions have to be used. These solutions contain different anions which by themselves may influence the I_{sc}. All the anions used (SO_4^{2-}, $CH_3SO_4^-$, and NO_3^-) gave similar results and thus, we felt secure in pooling the data. Toxicity of these anions is not likely in view of our observation that inhibition of I_{sc} upon substitution of Cl^- by either of these ions was always completely reversible within less than 1 min. 3. The concentration of ionized calcium, measured with an ion selective electrode varied slightly in the solutions (1.3–1.5 mmol $\cdot l^{-1}$). We have shown that changes of ionized calcium in this range of concentrations have no effect upon the measured I_{sc}. 4. We have used hyperosmolar solutions, and it may be inferred that this degree of hyperosmolality may have inhibited the I_{sc}. In fact, recently it was reported for the medullary TAL segment of the mouse that NaCl reabsorption was impeded by hyperosmolality [24]. We have shown a small significant inhibition of the I_{sc} by adding 300 mmol $\cdot l^{-1}$ mannitol to our control solution. It is worth noting that the mean I_{sc} for 300 mmol $\cdot l^{-1}$ in Fig. 1 is also slightly lower than that for 150 mmol $\cdot l^{-1}$. This difference (of 15%) is not statistically significant. 5. Most importantly, we treat the entire epithelium as if the ongoing ion transport were described by a simple chemical reaction. This treatment certainly is only appropriate if the limiting reaction is that at the lumen carrier and not that of the exit mechanism. We have no direct evidence in favour of this assumption. A pool size versus "flush through" [35] consideration for the cTAL segment reveals, however, that the turnover rate for the entire cellular content of Na^+ and of Cl^- is in the range of one time per s. In such a situation the cell would be constantly endangered by dramatic changes in intracellular composition if the lumen membrane would not be rate limiting, and if the cell would not have regulatory mechanisms to adjust both, lumen uptake and basolateral rate of Na^+ and K^+ pumping. In view of the above restrictions we feel that an apparent kinetics like that in Fig. 1 can only be used as circumstantial evidence for the stoichiometry of the cotransporter. Apart from these theoretical considerations, another problem arose when we started to analyse the data obtained in this series. It was clear from the beginning that the concentration dependence could be defined with the necessary accuracy only if as many different concentrations as possible were tested for each single tubule. In fact, in some of the tubules studied up to 8 different concentrations were tested. On the average, however, only 4 different concentrations were employed per tubule. Thus, it was not possible to obtain individual apparent kinetic constants for each single tubule and to average these constants for the entire series. Instead, the analysis was based on the sum of all data. We decided not to use the 3 classical plots to linearize the Michaelis-Menten equation because then a weighing factor would have been necessary to account for the bias introduced by each of the different plots. We resorted to a least square fit procedure which fits 3 parameters to the data: intercept, affinity term, and v_{max} term. With this computer-aided analysis it was possible to calculate the apparent kinetic constants with their respective standard error for the assumption that the data describe a Michaelis-Menten type of correlation between I_{sc} and $\{Cl^-\}^1$ concentration, between I_{sc} and $\{Cl^-\}^2$ concentration, and I_{sc} and $\{Cl^-\}^3$ concentration. As indicated in the results section assumption of a I_{sc}: $\{Cl^-\}^2$ correlation provides a statistically better fit of the data than the assumptions of a correlation between I_{sc} and $\{Cl^-\}^1$ and between I_{sc} and $\{Cl^-\}^3$. When comparing these results to those which we have obtained recently for the apparent kinetics for Na^+ [12] it is evident that there an optimal fit was obtained by assuming a simple Michaelis-Menten kinetics correlating I_{sc} to the $\{Na^+\}^1$ concentration, and the apparent affinity was considerably greater than reported here for Cl^-. This gives the explanation for the fact that all of the previous investigators were able to show that removal of Cl^- decreases the active transport in this nephron segment, yet none of these was able to detect the almost complete inhibition when Na^+ is removed [5, 25, 32].

As stated above, the present finding of an apparent $\{Cl^-\}^2$ dependence is only regarded as circumstantial evidence for the tentative conclusion that 2 Cl^- are involved in the Na^+-Cl^--K^+ cotransport system of the lumen membrane. It is, however, of interest that preliminary observations in lumen membrane vesicles prepared from rabbit medullary TAL point to a 2 Cl^- per 1 Na^+ stoichiometry [9, 26]. In fact, in this recent study [26] also the requirement of K^+, which we have reported recently [16], could be documented.

Is the Cotransporter System in the Lumen Membrane Charged?

The second and third series of experiments of the present investigation were aimed at testing whether the cotransporter, by carrying a negative charge, was responsible for the transepithelial *PD*. At one time [14] we have speculated that this would give a ready explanation for the finding that furosemide abolishes the active transport *PD* across the epithelium so rapidly when added to the lumen perfusate. In fact, series 2 reinforces this finding by showing that the half-value time for abolishing the *PD* may be as low as <0.4 s. The availability of intracellular *PD* measurements in this preparation [13] has now made it possible to test this hypothesis directly. The data summarized in Fig. 4 indicate clearly that inhibition of the luminal cotransporter by furosemide leads to a rapid hyperpolarization of the basolateral membrane. This finding, although unexpected, is in qualitative agreement to a previous observation in the Amphiuma diluting segment [29, 31] and has been confirmed meanwhile in preliminary reports on the isolated perfused Amphiuma and salamander diluting segment [21, 33]. In our study, but not in the original study in the Amphiuma diluting segment, the hyperpolarization of the basolateral membrane (Δ 15 mV) exceeds the change in transepithelial *PD* which was some 6 mV. Thus, also the lumen membrane hyperpolarizes by some 6–10 mV. It appears legitimate to conclude that this finding of rapid hyperpolarization of the lumen membrane makes it very unlikely that the cotransporter in the lumen membrane is negatively charged. Then, a depolarization after furosemide should have been observed.

Clearly, the argument may be turned around, and we have to respond to the question whether the cotransporter is positively charged, as we have observed a rapid hyperpolarization of the lumen membrane after furosemide. On the basis of comparable observations in the diluting segment of the salamander, in fact, the existance of a positively charged

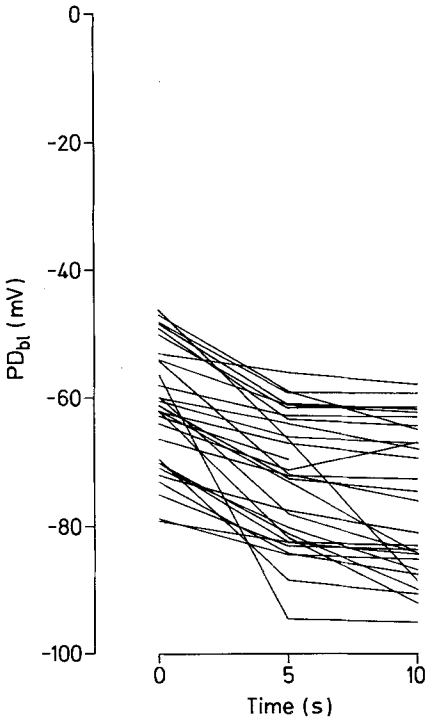

Fig. 4. Effect of furosemide ($1-5 \cdot 10^{-5}$ mol $\cdot l^{-1}$) on the *PD* across the basolateral membrane (PD_{bl}) of 30 cTAL segments. The mean hyperpolarization of PD_{bl} of 15 ± 2 mV is statistically significant

carrier has been postulated [33]. We believe that in the rabbit cTAL segment several lines of evidence make such an assumption very unlikely: 1. In the experiments depicted in Table 3 it was possible to measure the voltage divider ratio. The VDR, corresponding to the ratio of the lumen membrane resistance divided by that across the basolateral membrane, decreased significantly in the presence of furosemide, and this finding matches a recent observation in the Amphiuma diluting segment [21]. An increase in VDR, however, was expected if furosemide were to block a positively or negatively charged carrier contributing to the conductance of the lumen membrane. The observed fall in VDR after furosemide may be explained by the fall in intracellular Cl^- activity [19, 31] and a corresponding decrease in the Cl^- conductance of the basolateral membrane. 2. A positively charged carrier cannot explain the existance of the lumen positive PD_{te} as it carries depolarizing current across the lumen membrane, and makes it necessary to invoke opposing mechanism to explain the rapid collapse of the transepithelial *PD* after furosemide. 3. A ready explanation of the hyperpolarization after furosemide can be given if one makes the likely assumption that blockade of Cl^- entry into the cell by furosemide reduces intracellular Cl^- activity and that the basolateral membrane is Cl^- conductive. In fact, for both assumptions convincing evidence is present: As shown in a subsequent report [18] the basolateral membrane of the rabbit cTAL segment is conductive for Cl^-. Similar observations have also been made in the amphibian diluting segment [21, 29, 33]. In this preparation a dramatic fall in cell Cl^- activity after furosemide was clearly documented [31]. In a preliminary study [19] we have also utilized Cl^--selective electrodes, in the rabbit cTAL segment and were able to show that furosemide causes a rapid fall in intracellular Cl^- activity. Thus, we conclude that the hyperpolarization, observed after furosemide, is a secondary phenomena, caused most likely by the fall in intracellular Cl^- activity, and that the furosemide sensitive cotransporter in the lumen membrane operates electroneutrally.

Now we can return to the issue of what stoichiometry this cotransporter might have. More specifically, we may ask how many equivalents of K^+ are moved. Assuming the cotransport of 2 Cl^- and 1 Na^+ electroneutrality will be achieved by the inclusion of 1 equivalent of K^+. Independent evidence for the involvement of 1 K^+ comes from the observation that the K^+ current crossing the lumen membrane (calculated from the K^+ conductance of the lumen membrane and the electrochemical driving force for K^+ across this membrane [17]) is equal to the circular current. The latter corresponds to the movement of 1 equivalent. Complete recycling of K^+ across the lumen membrane then implies that also 1 equivalent of K^+ is taken up by the cotransporter. This issue of K^+ recycling across the lumen membrane of the cTAL segment will be subject of a subsequent report [17].

What is the Driving Force for the Electroneutral Cotransporter?

Finally, we have to answer the question of whether this type of cotransporter has a positive driving force, i. e. whether it will, in fact, move all 3 ion species from lumen to cell. To make this estimate the intracellular ion activities have to be known. We do have a reasonable estimate of the Cl^- activity within the rabbit cTAL cell which is in the order of $15-25$ mmol $\cdot l^{-1}$ [19]. An estimate of the K^+ activity can be obtained from the conductivity properties of the lumen membrane and estimates of the individual resistors which both are provided in a subsequent report [17]. In this study we arrive at an intracellular K^+ activity of some $90-100$ mmol $\cdot l^{-1}$. The sodium activity is not known. Taking the Na^+ concentration measured by electron probe in the distal tubule cell [2], and using an activity coefficient of $0.7-0.8$ we arrive at some 10 mmol $\cdot l^{-1}$ which is very similar to what has been reported for the amphibian diluting segment [29]. Using the estimates of the cellular activities for the three ions and using the corresponding activities in our Ringer like perfusion solution we calculate a positive driving force of some $50-80$ mV.

Comparison of the Cotransporter in the cTAL Segment to Other Systems

The fact that the furosemide sensitive cotransporter in the lumen membrane of the rabbit cTAL appears to be identical to that in a nonpolar cell such as the Ehrlich ascites tumor cell [10] and the erythrocyte [8, 22] poses the question of whether we are dealing with a rather general and fundamental process possibly present also in many other cells and epithelia. In fact, since our first description of this mechanism in the cTAL segment [16], exiting studies in other chloride transporting and furosemide sensitive epithelia have provided preliminary evidence that also in these structures K^+ is involved in the furosemide sensitive uptake step of Cl^- [3, 27, 30, 33]. Further studies are needed to test whether the stoichiometry is also identical in these epithelia. It is of course conceivable that the stoichiometry of this type of carrier is not fixed under all experimental conditions, or that alterations in the stoichiometry are part of a regulatory mechanism. So far we do not have any evidence in this regard for the cTAL segment.

For the Ehrlich ascites cell a varying stoichiometry has been postulated recently [1].

Acknowledgement. The authors gratefully acknowledge the technical assistance by Ms. C. Weidtke. We also want to thank Prof. Dr. E. Frömter and Prof. Dr. K. J. Ullrich for the continuous discussions.

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Received June 24 / Accepted December 13, 1982